2008

Sex Determination in Chelydra

Fredric J. Janzen
Iowa State University, fjanzen@iastate.edu

Sex Determination in Chelydra

Abstract
T HE SEX OF AN INDIVIDUAL is a fundamental trait, determining (in animals) whether sperm or eggs are transmitted to form the next generation and thus the pattern of genetic contribution. At the same time, a panoply of behavioral, physiological, and morphological traits intrinsically linked to gonadal sex shape the specific phenotypes of individuals and hence the dynamics of populations. Furthermore, mechanisms of sex determination greatly influence the primary sex ratio and potentially the population sex ratio and effective population size, which are important ecological and evolutionary parameters. Indeed, Fisher (1930) demonstrated that under most circumstances 1:1 primary sex ratios are expected and sexdetermining mechanisms that produce such balanced sex ratios should be favored by selection (see also Bull 1983).

Disciplines
Ecology and Evolutionary Biology

Comments
Janzen, Fredric. Sex determination in Chelydra . Pp. 146-157. 2008 The Johns Hopkins University Press. Reprinted with permission of Johns Hopkins University Press.

14

Sex Determination in *Chelydra*

FREDRIC J. JANZEN

THE SEX OF AN INDIVIDUAL is a fundamental trait, determining (in animals) whether sperm or eggs are transmitted to form the next generation and thus the pattern of genetic contribution. At the same time, a panoply of behavioral, physiological, and morphological traits intrinsically linked to gonadal sex shape the specific phenotypes of individuals and hence the dynamics of populations. Furthermore, mechanisms of sex determination greatly influence the primary sex ratio and potentially the population sex ratio and effective population size, which are important ecological and evolutionary parameters. Indeed, Fisher (1930) demonstrated that under most circumstances 1:1 primary sex ratios are expected and sex-determining mechanisms that produce such balanced sex ratios should be favored by selection (see also Bull 1983).

For these fitness reasons (and simply based on fair meiotic segregation of chromosomes), one might expect sex determination governed by sex chromosomes to be ubiquitous in dioecious organisms. Remarkably enough however, a wide array of taxa exhibit diverse sex-determining mechanisms, including environmental sex determination wherein the sex of an individual is determined permanently by one or more factors after conception (Bull 1983; Korpelainen 1990). This unusual sex-determining mechanism occurs perhaps most commonly as temperature-dependent sex determination (TSD) in many vertebrates, mainly "reptiles" (Bull 1983; Janzen & Paukstis 1991a). The frequency of TSD raises the question of why so many organisms would leave the important trait of sex determination to the vagaries of the environment.

To resolve this ultimate enigma properly requires clear answers to numerous ancillary questions concerning the basic biology of TSD. Such questions include the following: (1) When and how many times has TSD evolved? (2) When does tempera-

ture act developmentally to influence sex? (3) What temperatures produce which sex? (4) How do thermal reaction norms of sex ratio (or responses to them) vary intra- and interspecifically? (5) How does TSD work in nature? (6) What are the underlying physiological and molecular mechanisms? (7) Does temperature influence traits other than gonadal sex in a sex-specific manner? The answers to these questions will also illuminate the conservation implications of TSD under global climate change and human habitat modification, especially given that many species with TSD are already imperiled (Gibbons et al. 2000). Many species have served as subjects for studies of TSD, but common snapping turtles (*Chelydra serpentina*) have played a prominent role in many ways. This chapter will review our current understanding of sex determination in *C. serpentina*, describe key published contributions made to a broader understanding of TSD by research on this species, and suggest promising future directions for investigating TSD using *C. serpentina*.

Temperature-dependent sex determination in vertebrates was first documented clearly in an agamid lizard in a relatively obscure French-language publication more than 35 years ago (Charnier 1966); not long thereafter, this work was extended to testudinid and emydid turtles (Pieau 1971 and numerous pioneering publications since). Common snapping turtles were the fourth vertebrate species for which TSD was described (Yntema 1976). TSD has even been documented in fish (e.g., Conover 1984). Indeed, TSD has now been identified, primarily by laboratory studies, in a diversity of vertebrates including a few fish, some lizards, many turtles, and all tuatara and crocodilians (Bull 1980, 1983; Conover 1984; Ewert & Nelson 1991; Janzen & Paukstis 1991a; Ewert et al. 1994; Lang & Andrews 1994; Viets et al. 1994; Cree et al. 1995). In general, embryonic exposure to specific thermal conditions during a limited period of development (the temperature-sensitive period, TSP) causes male or female differentiation. Few individuals with indeterminate (i.e., incompletely differentiated) or intersex (i.e., a testis and an ovary) gonads are born and there is no evidence that individuals with TSD change sex at a later ontogenetic stage (e.g., Bull 1987). Despite the overall similarity in timing of the TSP, the patterns of TSD vary among vertebrate species. For example, European pond turtles (*Emys orbicularis*) produce female offspring at high incubation temperatures and male offspring at low ones (Pieau 1971; pattern Ia); just the opposite pattern seems to occur in the lizard *Agama agama* (Charnier 1966; pattern Ib). Furthermore, many taxa including *C. serpentina* produce female offspring at high and low incubation temperatures and male offspring at intermediate ones (Yntema 1976; pattern II). Even so, species with the same pattern of TSD may differ dramatically in the pivotal temperature of sex determination (T_{piv} is a temperature at which a 1:1 sex ratio is obtained). To illustrate, the upper T_{piv} of the Amazonian freshwater turtle (*Podocnemis expansa*) in Colombia is 32.6°C (Valenzuela 2001a), whereas the upper T_{piv} of *C. serpentina* in North America is approximately 5°C lower (Ewert et al. 1994). In fact, *C. serpentina* eggs do not even hatch at 32.6°C (Yntema 1978)! The ecology, physiology, molecular biology, and genetics of TSD in vertebrates are more poorly known, so general discussion of these elements will be deferred to the appropriate sections of this chapter.

ORIGIN OF TSD

Given the wide distribution of TSD in vertebrates and the documented great ages of the various groups in which TSD occurs, this sex-determining mechanism is almost certainly very old. But just how old? And did it evolve only once? Since TSD apparently does not leave any trace in the fossil record, the second question is somewhat easier to answer. TSD occurs in at least one species of teleost fish, but is not known in any amphibian, mammal, bird, or snake. This phylogenetic discontinuity almost certainly indicates that TSD has evolved at least twice, once in fish and once in "reptiles." But has TSD evolved more than once in amniotes? This question is highly debatable, but phylogenetic comparative analyses most parsimoniously suggest a single ancestral origin in amniotes and several more recent origins in lizards (Janzen & Krenz 2004). Given the antiquity of the split of "reptile stock" from mammals, the initial origin of TSD in amniotes is ancient (Kumar & Hedges 1998).

All species within the Chelydridae (*Chelydra* and *Macroclemys*) have TSD (Bull 1980; Janzen & Paukstis 1991a). Modern phylogenetic analyses place this family as a close relative to kinosternid turtles (Krenz et al. 2005), with the origin of the clade containing chelydrids being about 85 million years ago (Near et al. 2005). Consequently, TSD has no doubt long accompanied this lineage on its remarkably successful colonization of a diversity of freshwater habitats across a considerable latitudinal gradient from Ecuador to Canada (Ernst et al. 1994). How TSD in snapping turtles has adapted to the concordantly dramatic change in thermal environments across this large geographic range is unknown (but see discussion in "Evidence for TSD" below).

EMBRYOLOGY OF TSD

Perhaps no one has contributed more to our understanding of sex determination in *Chelydra* than Chester Yntema, who discovered TSD in this species more than 30 years ago (Yntema 1976). Indeed, Yntema's pioneering work on the embryology of TSD in *Chelydra* laid an incredibly solid foundation for subsequent research on numerous aspects of TSD in other species as well. Yntema's contributions are particularly notable for identifying the embryonic stages during which gonadal sex is sensitive to temperature (i.e., the TSP) (Yntema 1979) and for clearly illustrating morphological and histological features of the gonads (Yntema 1981). Similar experiments have been conducted to estimate the TSP in other turtles, a lizard, and several crocodilians, but have been far less extensive (reviewed in Janzen & Paukstis

1991a). Even fewer studies have provided detailed information on gross morphology and ultrastructure of gonads and accessory structures. Our understanding of the biological implications of TSD in species without such basic information often rests on the extent to which Yntema's yeomanly embryological work on *Chelydra* can be extrapolated.

Analogous to the famed chicken embryonic series (Hamburger & Hamilton 1951), Yntema developed a valuable embryonic series of stages for *Chelydra* (Yntema 1968). This staging work provided a common language of embryonic development and thereby permitted comparative embryological work at different incubation temperatures. Using this framework, Yntema (1979) showed in a series of exhaustive (and no doubt exhausting!) temperature-shift experiments that the TSP for *Chelydra* occurred approximately between developmental stages 14 and 19 (i.e., from when the forelimb is a simple paddle with a vague digital plate to when digits significantly protrude beyond the edge of the digital plate). Incubation temperatures prior to and subsequent to these developmental stages did not influence sex determination. This TSP corresponds roughly to the second one-fourth of embryonic development (Yntema 1968), which lasts about 1–3 weeks depending on temperature (Yntema 1979), although most turtle researchers have instead referred to this period as the middle one-third (e.g., Janzen & Paukstis 1991a). The TSP was much shorter developmentally (about stages 14–16), but perhaps not temporally, for both sexes when eggs were shifted to an exceptionally cool temperature (20°C) (Yntema 1979). Overall, the developmental stages of the TSP for *Chelydra* are roughly comparable to those of other turtle species examined, although the latter often appear to have a slightly extended TSP to stage 21 or 22 (reviewed in Janzen & Paukstis 1991a).

Equally important has been Yntema's excellent description and plates of gonadal morphology and accessory structures (Yntema 1976) as well as gonadal ultrastructure via histological examination (Yntema 1981). This information has no doubt initially guided numerous researchers studying TSD in amniotes because of its high quality and clarity! Yntema (1976) showed that a neonatal ovary is elongate with follicles present in the cortex, providing a bumpy exterior surface, and is accompanied laterally by a white, threadlike oviduct. In contrast, the neonatal testis is shorter and less elongate, with a smooth surface and no (or only a vestigial) oviduct. Observations on the gonads of unpreserved *Chelydra* neonates indicate that ovaries can be further recognized by their whitish hue, in contrast to the yellowish color and what appear to be transverse capillaries evident in testes (pers. observ.). Yntema's (1981) detailed histological examinations completely confirmed assignment of sex based on assessment of gross gonadal morphology. Testes were covered by a thin squamous epithelial layer and were packed with seminiferous tubules, whereas ovaries were covered by a layer of cuboidal cells and were filled with primary follicles (Yntema 1981). Although ovaries of hatchlings from 20°C and 30°C were similar in length and follicle density, the 20°C ovaries contained less developed germinal epithelium and a higher incidence of epithelial cysts than 30°C ovaries. This observation raises the question of whether individuals of the same sex produced at different incubation temperatures might have different fitnesses in the short term (e.g., Janzen 1995) or in the long term (e.g., Gutzke & Crews 1988).

EVIDENCE FOR TSD

Laboratory Incubation at Controlled Temperatures in Environmental Chambers

The first and foremost evidence for TSD and patterns of TSD derives from laboratory studies in which eggs are incubated at constant temperatures for most or all of embryonic development (Bull 1980; Ewert & Nelson 1991; Janzen & Paukstis 1991a; Ewert et al. 1994; Lang & Andrews 1994; Viets et al. 1994). Such data are particularly abundant for *Chelydra* and originate from several investigators and localities (Table 14.1) (Ewert et al. 2005). Plotting offspring sex ratio (% male) against incubation temperature for the combined data set reveals that *Chelydra* clearly has TSD; some temperatures produce 100% males and others produce 100% females (Fig. 14.1). These results are not caused by embryonic mortality (e.g., see analysis in Paukstis & Janzen 1990). The graphical analysis also reveals that *Chelydra* overall has pattern II TSD, with an average lower T_{piv} at ~21.4°C and an average upper T_{piv} at ~27.8°C. (These values were calculated using inverse prediction after logistic regression analysis of sex ratio data from Table 14.1 for 21 constant incubation temperatures from 20–24.5°C and 54 constant incubation temperatures from 23–31°C, respectively [SAS Institute 2000]). Temperatures below ~21.4°C and above ~27.8°C produce primarily female offspring; males are mostly produced at temperatures between ~21.4 and ~27.8°C.

Figure 14.1 further illustrates a robust fit of the data that might superficially suggest relatively little geographic variation in thermal sensitivity of sex determination in *Chelydra*. Indeed, the upper T_{piv} seems to exhibit some positive latitudinal trend in *Chelydra*, but the variation across ~30 degrees latitude is at most 2–3°C (Ewert et al. 1994). However, a comprehensive study reveals that such seemingly subtle geographic variation in TSD in *Chelydra* is, in fact, both statistically and biologically significant (Ewert et al. 2005). Populations of *Chelydra* arrayed across ~20 degrees latitude exhibit an inverse correlation between lower and upper T_{piv} values that accords with both local climate data and the nesting behavior observed in each population.

This relative invariance of the midrange of the male-producing range of incubation temperatures in *Chelydra* (Ewert et al. 2005) might cast doubt on the traditional utility of T_{piv} as a concept. Perhaps our efforts would, for example,

Table 14.1

Summary of published sex determination data from laboratory constant-temperature incubation studies of *Chelydra*

Males (*N*)	Females (*N*)	Intersexes (*N*)	% Male	Incubation temperature (°C)	Locality	Reference
			84	21.5	MN	Rhen & Lang 1998
32	2	0	94.1	22.5	MN	Ewert & Nelson 1991
134	0	0	100	24	MN	Rhen & Lang 1994
			100	24	MN	Rhen & Lang 1999a
130	1	0	99.2	26.5	MN	Rhen & Lang 1994
8	0	0	100	26.5	MN	Rhen & Lang 1996
			98	26.5	MN	Rhen & Lang 1999a
31	95	0	24.6	29	MN	Rhen & Lang 1994
10	40	0	20.0	29	MN	Rhen & Lang 1994
1	10	0	9.1	29	MN	Rhen & Lang 1996
			36	29	MN	Rhen & Lang 1999a
			24.6	29	MN	Rhen & Lang 1999b
5	22	0	18.5	22.0	no. ON	Brooks et al. 1991
9	0	0	100	25.6	no. ON	Brooks et al. 1991
3	6	0	33.3	28.6	no. ON	Brooks et al. 1991
52	197	0	20.9	20.9	ON	Bobyn & Brooks 1994b
16	85	0	15.8	21.3	ON	Bobyn & Brooks 1994a
51	11	0	82.3	24.8	ON	Bobyn & Brooks 1994a
150	4	0	97.4	25.1	ON	Bobyn & Brooks 1994b
1	42	0	2.3	29.1	ON	Bobyn & Brooks 1994a
0	58	0	0	20	NY/WI	Yntema 1976
0	149	0	0	20	NY/WI	Yntema 1981
19	2	0	90.5	22	NY/WI	Yntema 1976
18	0	0	100	24	NY/WI	Yntema 1976
108	0	0	100	26	NY/WI	Yntema 1976
79	2	0	97.5	26	NY/WI	Yntema 1976
373	3	0	99.2	26	NY/WI	Yntema 1981
17	9	0	63.0	28	NY/WI	Yntema 1976
0	87	0	0	30	NY/WI	Yntema 1976
0	142	0	0	30	NY/WI	Yntema 1981
54	0	0	100	23	NE	Paukstis & Janzen 1990
33	3	0	91.7	25	NE	Gutzke & Bull 1986
30	0	0	100	25	NE	Crews et al. 1989
102	1	0	99.0	26	NE	Packard et al. 1987
36	2	0	94.7	26	NE	Gutzke & Chymiy 1988
50	0	0	100	26	NE	Janzen et al. 1990
1	100	0	1.0	28.5	NE	Packard et al. 1987
0	63	0	0	29	NE	Packard et al. 1984
0	37	0	0	30	NE	Crews et al. 1989
0	33	0	0	31	NE	Gutzke & Bull 1986
0	50	0	0	31	NE	Packard et al. 1987
36	28	0	56.3	21.5	IL	O'Steen 1998
16	4	0	80.0	21.5	IL	O'Steen & Janzen 1999
9	12	0	42.9	21.5	IL	O'Steen & Janzen 1999
154	45	1	77.3	21.8	IL	Janzen et al. 1998
45	0	0	100	24.5	IL	O'Steen 1998
23	0	0	100	24.5	IL	O'Steen & Janzen 1999
36	0	0	100	26	IL	Janzen 1995
50	66	0	43.1	27.5	IL	Janzen 1992
47	47	0	50	27.5	IL	O'Steen 1998
20	24	0	45.5	27.5	IL	O'Steen & Janzen 1999
12	9	0	57.1	27.5	IL	O'Steen & Janzen 1999
91	104	2	46.7	27.6	IL	Janzen et al. 1998
46	178	1	20.7	28	IL	Janzen 1992
37	23	0	61.7	28	IL	Janzen 1995
9	108	0	7.7	28.5	IL	Janzen 1992
0	25	0	0	30	IL	Janzen 1995
0	36	0	0	30.5	IL	O'Steen 1998
0	15	0	0	30.5	IL	O'Steen & Janzen 1999
2	6	0	25.0	21.5	IN	Ewert & Nelson 1991
5	5	0	50.0	22.5	IN	Ewert & Nelson 1991

continued

Table 14.1 continued

Males (*N*)	Females (*N*)	Intersexes (*N*)	% Male	Incubation temperature (°C)	Locality	Reference
7	1	0	87.5	26	DC/VA/MD	Dimond 1983
3	10	0	23.1	28.5	DC/VA/MD	Dimond 1983
0	13	0	0	31	DC/VA/MD	Dimond 1983
0	3	0	0	21.5	TN	Ewert & Nelson 1991
11	2	0	84.6	25	MX	Vogt & Flores-Villela 1992
4	29	0	12.1	28	MX	Vogt & Flores-Villela 1992
0	3	0	0	29	MX	Vogt & Flores-Villela 1992
0	2	0	0	30	MX	Vogt & Flores-Villela 1992

Entries are arranged by population first by descending approximate latitude, then by ascending incubation temperature within each general locality, followed by ascending year of publication within a given incubation temperature.

be more profitably spent on characterizing the shapes of the entire reaction norms of temperature and sex ratio rather than just on analyzing these one or two single T_{piv} values within the reaction norms (sensu Girondot 1999). To illustrate, walking speed (think of this as T_{piv}) is identical between a Congolese and a French population of *Drosophila melanogaster*, but developmental temperature and population exhibit a significant interaction effect (i.e., the shape of the reaction norm for walking speed differs between the two populations; Gibert et al. 2001). Without identifying the interaction, one might logically conclude that walking speed was similar in the two populations. Instead, the interaction reveals that flies from France walked fastest if derived from an 18°C developmental temperature compared to 25 or 29°C, and flies from the Congo walked fastest if derived from a 25°C developmental temperature compared to 18 or 29°C (Gibert et al. 2001). These results suggest local adaptation to different natural thermal environments that would have been obscured by simply focusing on one point (i.e., the mean) of the reaction norm.

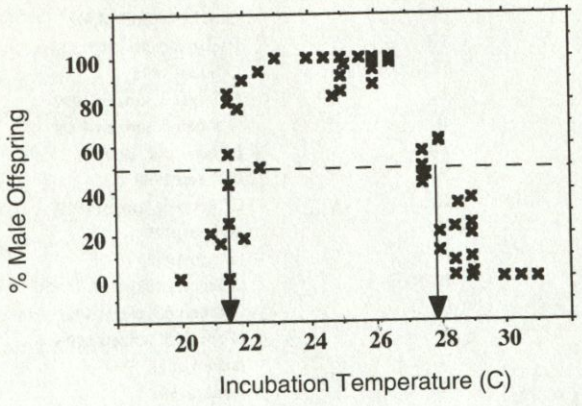

Fig. 14.1. Percent male offspring produced from laboratory constant-temperature incubation studies of *Chelydra*. The graphic of 69 data points (Table 14.1) illustrates that *Chelydra* has pattern II TSD, with a fairly narrow range of lower and upper temperatures tending to produce mixed sex ratios. An even offspring sex ratio (dashed line) is expected at all temperatures in species with genotypic sex determination. Arrows indicate the approximate lower and upper T_{piv}s for *Chelydra*.

In contrast to the plethora of constant-temperature experiments, only two published laboratory studies involving *Chelydra* have even begun to address the effects of controlled diel fluctuations in temperature on sex determination. Fluctuation between 28 and 31°C (12 h at each temperature) produced only females among the offspring sexed from twelve clutches from Illinois (Kolbe & Janzen 2001). The more important contribution in this regard investigated the effects at different exposure times from 22 or 24°C to 30°C in *Chelydra* eggs from New Jersey (Wilhoft et al. 1983). Eggs that spent 4, 8, or 10 h/day at 30°C, and the remainder at 22°C, yielded 100% female offspring; eggs that spent 1, 2, or 3 h/day at 30°C, and the remainder at 24°C, yielded 9.0, 16.6, and 33.3% female, respectively. Thus, only 3–4 h/day incubation at 30°C (with the remainder at a male-producing temperature) tips the balance in favor of female-biased offspring sex ratios. The paucity of such experiments involving *Chelydra* is surprising because major diel fluctuations in temperature characterize the embryonic environment in natural nests (e.g., Packard et al. 1999). As described below, more experiments are critically needed to inform us on the sex-determining relationship between fluctuating temperatures that characterize natural nests and constant temperatures used in most laboratory studies.

Field Incubation at Fluctuating Temperatures in Natural Nests

One of the trickier issues regarding TSD since its discovery concerns its operation under natural conditions. This knowledge is crucial for interpreting some of the most important ecological, evolutionary, and conservation implications of TSD. Does TSD occur in nature or is it a laboratory artifact? How, if at all, do fluctuating temperatures lead to predictable offspring sex ratios? Is pattern II TSD ecologically relevant? Do changing climates alter offspring sex ratios?

The earliest work on TSD in natural nests involved *Emys orbicularis* (e.g., Pieau 1974), yet the first comprehensive research to show clearly that TSD works in nature involved *Chelydra* (Wilhoft et al. 1983; Bull & Vogt 1979; and see be-

low). Since then, a variety of field studies of TSD have been conducted, only a few of which have involved actual experimentation or have measured nest temperatures continuously (also see older literature reviewed in Janzen & Paukstis 1991a). These studies confirm the early field work: TSD occurs in natural nests of many species, as indicated by bimodal distributions of nest sex ratios, and roughly conforms to expected patterns based on laboratory studies.

Despite such results, the arithmetic mean of fluctuating temperatures in a given natural nest is not equivalent in its sex-determining effect to an identical constant incubation temperature used in a laboratory study. The thermal variance clearly matters too. Different statistical models have been successfully developed to equate fluctuating temperature regimes to constant temperature equivalents in the loggerhead sea turtle *Caretta* (Georges et al. 1994) and in *Podocnemis expansa* (Valenzuela 2001a; see also Bull 1985 for map turtles *Graptemys* and Schwarzkopf & Brooks 1985 for painted turtles *Chrysemys*), but these approaches have yet to be applied extensively to *Chelydra*. In fact, their overall utility for understanding the ecology of TSD remains to be shown, but the idea of generating sophisticated statistical/mathematical models holds great promise.

The few published field studies of TSD in *Chelydra* are especially illustrative of the great need for additional work on various issues in this area. Wilhoft et al. (1983) monitored offspring sex ratios and temperatures in eight "reconstructed" *Chelydra* nests in New Jersey. They also recorded offspring sex ratios, but not temperatures, in four natural nests. Sex ratios varied from 100% male to 100% female in the "reconstructed" nests; a similar result was noted for the natural nests. Moreover, in nests with mixed sex ratios, more females were produced in the upper (warmer) levels than in the lower (cooler) levels. These results highlight the potential impact on offspring sex ratios of temperature gradients in the deeper nests of larger species like *Chelydra*.

More recently, Kolbe & Janzen (2002) analyzed data provided in Packard et al. (1999) for 15 *Chelydra* nests in Nebraska for which offspring sex ratios and temperatures (8 weeks at 30-min intervals) at the bottom of nests were recorded. Sex ratios (% males) were negatively correlated with the reported mean temperatures ($r = -0.47$, $P = 0.07$). Similarly, Kolbe & Janzen (2002) detected a negative correlation between mean nest temperatures (middle one-third of development at 72-min intervals) and sex ratio of 10 randomly chosen offspring from each of 14 *Chelydra* nests in Illinois ($r = -0.73$, $P = 0.003$). This finding was mostly confirmed in a smaller-scale study in the same population in a subsequent year (St. Juliana et al. 2004).

These studies illustrate several key points about the ecology of TSD. Note, in particular, that no field study detected convincing evidence of female offspring at lower incubation temperatures, calling into question the ecological relevance of pattern II TSD in *Chelydra* (see Valenzuela [2001a] for a similar conclusion for *P. expansa*). Perhaps *Chelydra effectively* has pattern Ia TSD under natural conditions (see also Ewert et al. [2005] for a similar conclusion). Second, compared with laboratory temperatures, mean nest temperatures do a poor job of explaining variation in sex ratios (≤50%) (see also discussion above). This problem is confirmed by comparing the predicted upper T_{piv} values (±1 SD) from these field studies (field T_{piv} IL = 25.46 ± 0.30°C vs. field T_{piv} NE = 25.40 ± 0.08°C [excluding the one cold nest because it may, in fact, have involved the lower T_{piv}]) with those calculated for these two populations based on laboratory research (lab T_{piv} IL = 27.52 ± 0.03°C [14 data points from Janzen 1992, 1995; Janzen et al. 1998; O'Steen 1998; O'Steen & Janzen 1999] vs. lab T_{piv} NE = 27.11 ± 0.11°C [11 data points from Packard et al. 1984, 1987; Gutzke & Bull 1986; Gutzke & Chymiy 1988; Crews et al. 1989; Janzen et al. 1990; Paukstis & Janzen 1990]), using the maximum likelihood method of Girondot (1999). The field T_{piv} values are sharply lower than the lab T_{piv} values; in fact, incubating eggs in the laboratory at the field T_{piv} values would produce all, or nearly all, male offspring (Fig. 14.1)! This disconnect between mean nest temperatures and constant temperatures clearly indicates the importance of fluctuating temperatures to natural sex determination in species with TSD.

And what of the impact of among-year (or longer-term) climatic variation on cohort sex ratios in amniotes with TSD? Few studies have addressed this question for obvious logistical reasons. The two most extensive investigations have documented clear and strong links between annual variation in local thermal climate and cohort sex ratios in *Caretta* (Mrosovsky & Provancha 1992) and in *Chrysemys* (Janzen 1994a). Furthermore, in both cases, the relationship between climatic temperatures and cohort sex ratios was clearly in accord with the relationship between constant incubation temperatures and offspring sex ratios observed in laboratory studies of these two species. Far less is known in *Chelydra*. Schwarzkopf & Brooks (1985), citing unpublished work, report that "a female bias (sex ratio = 0.31) [was detected] in hatchling snapping turtles from natural nests in Algonquin Park [Ontario, Canada] every year for 3 years." Similarly, Kolbe & Janzen (2002a) noted a heavily female-biased offspring sex ratio (~8.6% male) in 1999 in Illinois, as did St. Juliana et al. (2004) for the same population in 2001 (~22.1% male). These field studies were conducted in warmer-than-average years at the Illinois site (1999 was tied for the third warmest July and 2001 was tied for the eleventh warmest July in Clinton, Iowa, between 1951 and 2004). Much work remains to be done on this important topic, but these studies indicate that among-year variation in climatic conditions may play a substantial role in influencing cohort sex ratios.

MECHANISM OF TSD

Physiology and Endocrinology

One of the hot, and more intriguing, questions involving sex-determining mechanisms concerns the physiological

and molecular underpinnings of TSD. This area of research is receiving rapidly expanding attention. Still, we know little about the underlying mechanism(s) of TSD at this point. Much recent research on amniote TSD has focused on molecular bases and physiological pathways (see Lance 1997 and Pieau et al. 1999 for excellent reviews).

Snapping turtles have served as one of the primary models for early research in this area. Injecting or topically treating *Chelydra* eggs near the beginning of the TSP with sex steroids, agonists, antisera, or enzyme inhibitors has revealed important insights into the endocrinological aspects of TSD. One critical result illustrated good concordance between the TSP and the developmental period when differentiating gonads are sensitive to exogenous hormones (Gutzke & Chymiy 1988). This work set the stage for subsequent manipulative endocrinological experiments to ascribe physiological relevance to their results and suggested a causal role for sex steroids in gonadal differentiation. In another experiment, eggs treated with testosterone and then incubated at a female-producing temperature (31°C) yielded no male offspring; interestingly, testosterone-treated eggs incubated at a male-producing temperature (25°C) yielded a preponderance of females (Gutzke & Bull 1986). Furthermore, estradiol completely overrode temperature effects on sex determination at 25°C, leading only to female offspring. These results were largely confirmed in subsequent studies (Crews et al. 1989; Rhen & Lang 1994).

These latter two studies further defined the impact of estrogens on female differentiation in *Chelydra*. Treating eggs at a mostly female-producing temperature (29°C) with an aromatase inhibitor (aromatase converts testosterone to estradiol) had a masculinizing effect (Rhen & Lang 1994). Topical application of estradiol to eggs elevates gonadal aromatase activity at a primarily male-producing temperature (26.5°C), but no information is available to judge this effect at a female-producing temperature (Place et al. 2001). Moreover, Crews et al. (1989) showed that a synthetic estrogen agonist completely overrode temperature effects on sex determination at a male-producing temperature (25°C), concluding that "the classical high affinity, low capacity estrogen receptor" mediates this feminizing effect. Although this information identifies an important, perhaps causal, role for estrogens and aromatase in sex determination of amniotes with TSD, note that administering estradiol to eggs of "reptiles" with genotypic sex determination (GSD) also results in only female offspring (Bull et al. 1988b).

Crews et al. (1989) concluded that androgens are unlikely to be testis-inducing substances because they had negligible effects on sex determination at a female-producing temperature (30°C) (see also Rhen & Lang 1994). These results contrast with an intriguing, albeit weak, positive correlation observed between the concentration of testosterone in yolk at oviposition and the frequency of male offspring produced for 20 clutches of *Chelydra* eggs from Illinois (Janzen et al. 1998). The reason for this difference may reside in the fact that Janzen et al. (1998) incubated eggs near the upper T_{piv} (27.6°C), which engenders greater sex-determining sensitivity than more extreme temperatures (e.g., Wibbels & Crews [1995] for sliders *Trachemys*). Indeed, lack of expression of gonadal aromatase is insufficient alone for testis development (Place et al. 2001), leaving open the possibility that some androgenic substances may be functionally involved.

As suggested above, recent research has detected considerable concentrations of testosterone and estradiol in egg yolks of turtles at oviposition (Janzen et al. 1998; Elf et al. 2002a) and these substances have been implicated in influencing offspring sex ratio in *Chrysemys* in the laboratory at the T_{piv} (Bowden et al. 2000). For *Chelydra*, yolk testosterone concentrations differed markedly among clutches and less so among the four eggs assayed for each clutch (see fig. 2 in Janzen et al. 1998; Elf et al. 2002a). Yolk estradiol levels for *Chelydra* were low and relatively uniform within and among clutches in the Illinois population (Janzen et al. 1998), but exhibited greater among-clutch variation in a Minnesota population (Elf et al. 2002a). The two turtle species examined by Janzen et al. (1998) with GSD had low, fairly constant levels of testosterone in egg yolks. Although yolk testosterone concentrations for *Chelydra* were positively correlated with the frequency of male offspring near the upper T_{piv} (27.6°C), this relationship did not approach significance in the vicinity of the lower T_{piv} (21.8°C). Moreover, the clutch sex ratio of hatchlings produced from eggs incubated at 28.4°C in this *same* population was unrelated to the concentration of testosterone (or estradiol) measured in the egg yolks (St. Juliana et al. 2004). Thus the biological importance of the positive testosterone result reported by Janzen et al. (1998) is questionable. Similarly, Elf et al. (2002b) noted that estradiol, but not testosterone levels were higher in yolks of embryos developing at warmer incubation temperatures than in yolks of embryos developing at cooler incubation temperatures. In apparent contrast to the lack of relationship between yolk estradiol and clutch sex ratio reported by St. Juliana et al. (2004), this result suggests that yolk estradiol might indeed play an important role in embryonic sexual differentiation in *Chelydra*, as it does in *Chrysemys* (Bowden et al. 2000). Our understanding obviously could benefit greatly from evaluating these endocrinological factors in additional populations, under controlled fluctuating temperatures in the laboratory, and under natural conditions. Future research on clarifying the importance of these maternal physiological effects on sex determination in species with TSD offers great promise.

Overall, these endocrine results have been cleverly coopted to address other important issues regarding TSD in amniotes (see "Adaptive Significance" below). To be specific, the effects of temperature and sex on potentially fitness-related traits have been investigated by experimentally generating offspring of the opposite sex at otherwise "single sex"–producing temperatures (e.g., Rhen & Lang 1995). Further major advances in clarifying the physiology of TSD are likely to await advances on the molecular front.

Molecular Biology

The molecular basis of GSD in amniotes is increasingly evident (e.g., Ramkissoon & Goodfellow 1996), and our understanding will surely benefit greatly from recent advances in obtaining genomic DNA sequences. In contrast, our knowledge of the molecular basis of TSD currently constitutes a gaping black hole: little has been illuminated and an expanding number of workers are being attracted (inescapably?) to the issue. Indeed, one of the experimental benefits of examining the molecular biology of sex determination by using species with TSD is that individuals with *essentially identical genetic compositions* can be environmentally induced to become male or female. My coverage of this topic will necessarily be brief both because of the vast lacunae in our knowledge base and because of excellent recent reviews (e.g., Pieau et al. 1999; Place & Lance 2004).

Early evidence deriving from mechanistic studies of TSD in "reptiles" has implicated steroid hormones in sexual differentiation of gonads, thus much molecular research has focused on the enzymes responsible for steroidogenesis (reviewed in Lance 1997; Pieau et al. 1999). Of particular interest has been aromatase, which is critical for ovarian differentiation and maintenance. Elevated amounts (but not activity levels) have been detected in gonads of *Emys* embryos from eggs incubated at female-producing temperatures compared with those incubated at male-producing temperatures (Pieau et al. 1999). Accordingly, cloning and expression studies have implicated differential regulation of the aromatase gene in female and male gonads at the level of transcription (Pieau et al. 1999). Of course, other genes are also necessary in such a sexual differentiation cascade. Future research on the molecular genetics of TSD would do well to examine them simultaneously to obtain a complete picture, in particular, if they act upstream of aromatase (e.g., steroidogenic factor 1; Fleming & Crews 2001), and to adopt a rigorous comparative approach in assaying key taxa with and without GSD.

The only published research on the molecular genetic underpinnings of TSD involving *Chelydra* examined tissue of neonates from New Jersey eggs incubated at female-producing (30°C) and male-producing (26°C) temperatures for ZFY- and SRY-like genes (Spotila et al. 1994). The latter genes (but not the former: Bull et al. 1988c; Koopman et al. 1989) have been implicated in the sex-determining pathway for males in all mammals examined (e.g., Sinclair et al. 1990). The turtle ZFY homologue (called Zft) was highly similar to mammal ZFY (167 of 180 amino acids) and even more so to chicken and alligator homologues. Conversely, probes from SRY would not hybridize to turtle DNA under high stringency, suggesting weak similarities. Focusing just on the HMG-box region of SRY revealed multiple unique but related sequences with minimal similarity to mammalian SRY (57–70%). Overall then, an SRY-like gene is unlikely to be involved in TSD.

Despite minimal information at this point, it is difficult to imagine that the entire molecular underpinnings of TSD are fundamentally different from those of GSD, despite their strikingly varied manifestations and biological implications. This hypothesis of a largely conserved molecular genetic pathway underlying both GSD and TSD finds support in the frequency of evolutionary changes between the two categories of sex-determining mechanisms (Janzen & Paukstis 1991a, b) and in the phylogenetic homology of genetic elements in other fundamental traits (reviewed in Gerhart & Kirschner 1997), including in the developmental genetics of testicular differentiation (Smith et al. 1999). In the end, dissecting the molecular bases of TSD will clarify the simplicity or complexity of evolutionary transitions between TSD and GSD and between different types of TSD and could also very well illuminate a general framework for the molecular biology of environmentally regulated systems.

EVOLUTION OF TSD

Quantitative Genetics

Even with knowledge of the physiological and molecular mechanisms underlying TSD, we would still be left with an incomplete understanding of the evolutionary potential of TSD. This is the realm of microevolution. The first quantitative genetic model of TSD (Bulmer & Bull 1982) described its microevolution as being governed by selection on, and heritable variation for, thermal sensitivity of offspring sex determination (e.g., T_{piv}) and maternal choice of thermal qualities of nest sites. Although most empirical effort to date has focused on evaluating the microevolutionary potential of T_{piv} (see below), this model emphasized that nest-site selection might be the more important component (Bulmer & Bull 1982; but see Morjan 2003).

Evaluations of T_{piv} have focused either on (1) geographic variation (interpreted in terms of expected clines in environmental temperatures) or (2) within-population variation (usually interpreted in quantitative genetic terms). In the first case, any geographic variation would embody the collection of evolutionary forces acting on (drift, selection, etc.) or underlying (heritability, genetic covariances, etc.) the pattern (i.e., reaction norm) of TSD. Early studies detected little geographic variation in T_{piv} for *Caretta, Chrysemys, Trachemys,* and *Graptemys* (e.g., Bull et al. 1982b; Limpus et al. 1985; Mrosovsky 1988) and what little pattern existed tended to contradict perhaps naïve predictions from adaptationist theory. More recently, Ewert et al. (1994) summarized information on geographic variation in T_{piv} in various turtles with TSD. For eight populations of *Chelydra*, the upper T_{piv} increased positively with latitude ($r = +0.69$, $P < 0.05$), contradicting expectations from basic adaptationist theory (see also Ewert et al. 2005). This departure from expectation turns out to be explained well by geographic variation in nesting behavior. The nesting season begins earlier and females choose shadier microenvironments in which to nest in southern populations of *Chelydra* than in northern populations (Ewert et al. 2005). Thus, the thermal environment

experience by developing embryos is probably "cooler" in southern populations than in northern ones, consistent with the observation described above of a positive correlation between the upper T_{piv} and latitude. The lower T_{piv} in *Chelydra* exhibits an inverse correlation with latitude ($r = -0.98$, $P < 0.0001$), which reflects a strong inverse correlation with the upper T_{piv} (Ewert et al. 2005), possibly indicating a genetic covariance between the T_{piv} values.

Intrapopulation studies of T_{piv} have detected substantial (among-clutch) variation. As acknowledged in these studies, within-population variation in T_{piv} could be caused by nongenetic maternal effects (e.g., steroid hormone content of egg yolks at oviposition) rather than genetic effects, causing heritability estimates to be at the upper limits. This distinction is important because the evolutionary dynamics of TSD differ dramatically depending on the causal basis of within-population variation in T_{piv} (Morjan 2003).

Bull et al. (1982a) initially described among-clutch variation for *Graptemys* and interpreted it in quantitative genetic terms ($h^2 = 0.82$). Because their laboratory study was conducted at a constant incubation temperature, thereby minimizing environmental variation, they constructed a metric (i.e., the realized heritability) to estimate the quantitative genetic basis of T_{piv} in nature. Temperatures differ substantially among nests; thus, the realized heritability of T_{piv} was considerably smaller ($h^2 = 0.06$) than that of the laboratory T_{piv}, indicating a microevolutionary constraint on T_{piv} under natural conditions.

A similar outcome has been noted for *Chelydra*. Incubating eggs from Illinois at three temperatures near the upper T_{piv}, Janzen (1992) detected substantial among-clutch variation that was interpreted in quantitative genetic terms ($h^2_{27.5} = 0.60$, $h^2_{28.0} = 0.76$, $h^2_{28.5} = 0.34$, $h^2_{combined} = 0.56$); the corresponding realized heritabilities were much lower (0.05, 0.06, 0.03, and 0.05, respectively). Analysis of variance suggested no significant genotype by environment interactions (i.e., clutch sex ratio responses were concordant across temperatures), which was confirmed by high genetic correlations for sex between the temperature treatments ($r^2_{7.5\times28.0} = 0.73$, $r^2_{7.5\times28.5} = 0.52$, $r^2_{8.0\times28.5} = 0.67$). Parallel with the T_{piv} results, this finding suggests that the shape of the TSD pattern in *Chelydra* is also genetically constrained in its microevolutionary potential (see also among-population results described in Ewert et al. 2005).

This conclusion about the microevolutionary potential of TSD in *Chelydra* has been criticized on methodological and sample-size grounds. Incubating eggs from Minnesota at four temperatures near the upper T_{piv}, Rhen & Lang (1998) also detected substantial among-clutch heterogeneity in sex ratio at 27.5, 28.0, and 28.5°C (but not at 29.0°C where nearly all offspring were female). However, using a logistic model of analysis of variance, significant genotype by environment interactions were detected between 27.5 and 28.0°C and between 27.5 and 28.5°C; genetic correlations for sex for these two comparisons were correspondingly low, suggesting that TSD in *Chelydra* is *not* constrained in its microevolutionary potential. Reanalysis of Janzen's (1992) data using a logistic model of analysis of variance still did not detect a significant genotype by environment interaction ($P = 0.7045$) and using Rhen & Lang's (1998) method for calculating genetic correlations actually strengthens Janzen's original conclusions (cf. $r^2_{7.5\times28.0} = 0.79$, $r^2_{7.5\times28.5} = 0.60$, $r^2_{8.0\times28.5} = 0.83$)! As for sample size, Janzen (1992) used nine fewer clutches (15 vs. 24) than Rhen & Lang (1998), but apparently sexed equivalent or higher numbers of offspring for each clutch and temperature combination. Thus differences between the two studies might be due to different genetic architectures in each population or to relatively small sample sizes (≤6/clutch) at 27.5°C for the Minnesota population. Replicate studies in these same or in other populations of *Chelydra* might usefully clarify this important question.

Very little research has focused on nest-site selection in the context of TSD despite its central role in various models concerning the microevolution of TSD (Bulmer & Bull 1982; Roosenburg 1996; Reinhold 1998; Freedberg & Wade 2001). Until recently, evidence for geographic variation in nest-site selection was essentially anecdotal. For *Chelydra*, populations in the southeastern United States regularly nest in the "shade" (Richmond 1945; Ewert et al. 1994) and in southern Mexico often nest "in or beneath vegetation" (Vogt & Flores-Villela 1992), whereas populations in northern Illinois (Kolbe & Janzen 2002), New Jersey (Wilhoft et al. 1983), northern New York (Petokas & Alexander 1980), Quebec (Robinson & Bider 1988), and possibly southern Michigan (Congdon et al. 1987) and northern Minnesota (Rhen & Lang 1995) typically nest in relatively unshaded microenvironments. These natural history observations have been confirmed by a large-scale semiquantitative analysis of vegetation cover around *Chelydra* nests (Ewert et al. 2005): nests in southern populations are highly shaded and those in northern populations are nearly unshaded. More detailed comparative work (e.g., controlled reciprocal transplant experiments) would be helpful to distinguish between local adaptation and phenotypic plasticity as explanations for these patterns, but these studies nonetheless suggest the overriding importance of nest-site selection in affecting the embryonic thermal environment and thus its potential impact on the microevolution of TSD. These conclusions are strengthened by the documented relationships between nest temperatures and nest sex ratios in *Chelydra* (see "Evidence for TSD" above).

But how can females choose the nest thermal environment to possibly manipulate offspring sex ratio when the TSP begins several weeks after oviposition? Indeed, thermal conditions at oviposition can be influenced by numerous transient environmental factors (e.g., cloud cover, proximity to precipitation event, etc.) that may be unlinked to nest temperatures during the TSP. Several studies have noted a

qualitative relationship between degree of vegetation cover around nests and nest sex ratio (e.g., Vogt & Bull 1982, 1984) and others have implemented quantitative measures documenting the same result (e.g., Janzen 1994b). Extending this latter approach to *Chelydra*, Kolbe & Janzen (2002) demonstrated that females in an Illinois population overall selected less vegetated sites in which to nest more frequently than expected based on available habitat. Even so, females exhibited substantial heterogeneity in overstory vegetation cover around nests at oviposition, which was strongly negatively correlated with nest temperatures during the middle one-third of incubation ($r = -0.82$, $P = 0.0001$, $n = 16$) and thus with nest sex ratio ($r = +0.98$, $P < 0.0001$, $n = 14$) (see also St. Juliana et al. 2004). Vegetation cover at oviposition can therefore provide a relatively reliable cue to manipulate relative offspring sex ratio.

At the same time, the inheritance of nest-site selection is unclear. Laboratory analyses of geckos with TSD document relatively little among-female variation in nest-site selection in a thermal gradient (Bull et al. 1988a; Bragg et al. 2000). Multiyear field studies of nest-site selection in *Chrysemys* have detected significant individual repeatability for vegetation cover around nests at oviposition (Janzen & Morjan 2001; Valenzuela & Janzen 2001). Repeatability is crucial for a phenotype to be a meaningful target of natural selection and delimits the upper bound of heritability for the trait (see discussion in Janzen & Morjan 2001). Thus, these remarkable field repeatabilities suggest that TSD in *Chrysemys* exhibits microevolutionary potential, providing empirical support for key aspects of models involving nest-site selection. At present, however, we lack any information on the quantitative genetics of nesting biology of *Chelydra*. Creative field experiments, perhaps linking molecular markers and phenotypic variation (e.g., Ritland 1996), on any species with TSD would be extremely valuable for revealing the microevolutionary potential of nest-site selection.

Yet a third possible factor involved in the microevolution of TSD has been almost completely ignored so far. The growing number of studies of a wide variety of taxa has shown that reproductive phenology varies considerably with climatic conditions (e.g., Beebee 1995; Crick & Sparks 1999). Thus timing of the nesting season for amniotes with TSD may also be altered in response to geographic or annual variation in climate (e.g., Congdon et al. 1987; reviewed in Mrosovsky 1994). Indeed, Ewert et al. (2005) find that the nesting season begins and ends earlier in southern populations of *Chelydra* than in northern populations. If this response involves genetic change and not (just) behavioral plasticity, then adaptive evolution of reproductive phenology that potentially maintains offspring sex ratio variation may occur. This reasonable possibility deserves serious quantitative attention and will probably require a consortium of cooperative researchers to evaluate experimentally and over the long term.

Adaptive Significance

Of course, the million dollar question regarding TSD in amniotes is "Why does it exist or persist?" Why indeed do organisms with TSD leave the fundamental trait of sex determination to the stochastic whims of the environment? Despite much attention to this question over the past 30 years, a clear general answer remains elusive (Shine 1999).

The prevailing theoretical framework used to empirically assess the adaptive significance of environmental sex determination (Charnov & Bull 1977) has been successful in nonamniotes (mostly reviewed in Bull 1983; Conover 1984; Korpelainen 1990). The key component of this Charnov-Bull model is the expectation that environmental sex determination is adaptive when some environmental conditions (e.g., cooler incubation temperatures) are better (i.e., increase fitness) for males than for females and vice versa for other environmental conditions (e.g., warmer incubation temperatures) (for a graphical example, see Fig. 3 of Janzen 1995). In other words, the ratio of male to female fitness must vary with temperature in species with TSD. This expectation and theoretical modifications thereof (Roosenburg 1996; Reinhold 1998) have spawned several empirical studies of "reptiles" with TSD seeking thermally dependent traits that have sex-specific benefits (Shine 1999).

Research on *Chelydra* has contributed significantly to our body of knowledge on this topic. Most of this work has centered on the presumed sex-specific fitness advantages of differential posthatching growth rates (mostly summarized in Freedberg et al. 2001). The idea here is that faster growth particularly benefits males in *Chelydra* because they are the larger sex as adults and may defend home ranges (i.e., access to mates?) based on size (e.g., Janzen & O'Steen 1990). Thus, under this sexual size dimorphism hypothesis, males should be produced at incubation temperatures that elicit the fastest posthatching growth rates.

In general, the evidence favors this view. Turtles from Ontario grew fastest over 7 months from 25.6°C incubation as embryos (100% male) compared with turtles from 22.0°C (19% male) and 28.6°C (33% male) (Brooks et al. 1991). A similar result was obtained for four Ontario populations when grown for 11 months posthatching (i.e., 24.8°C turtles [82% male] grew faster than 21.3°C [16% male] and 29.1°C [2% male] turtles) (Bobyn & Brooks 1994a) and for two Ontario populations when grown for 23 months posthatching (i.e., 25.3°C turtles [97% male] grew faster than 21.1°C [21% male] turtles) (Bobyn & Brooks 1994b). Unfortunately, these studies could not separate the potential independent effects of sex and incubation temperature on posthatching growth.

Rhen and Lang adopted a clever method to do just that. By applying estradiol or an aromatase inhibitor to eggs during the TSP, they could generate females at otherwise male-producing temperatures or males at otherwise fe-

male-producing temperatures, respectively, that were presumably similar functionally to "normal" turtles of the same sex (Rhen et al. 1996). They found that Minnesota turtles from temperatures that normally produced exclusively males (24 and 26.5°C) grew faster over 6 months posthatching *regardless of sex* than turtles from the otherwise mostly female-producing temperature (29°C) (Rhen & Lang 1995, 1999b). Thus temperature, but not sex per se, influenced posthatching growth rates in accordance with the Charnov–Bull model. Unfortunately, they did not examine the effects of incubation temperature near or below the lower T_{piv}.

On the other hand, O'Steen (1998) incubated Illinois eggs at 21.5°C (56% male), 24.5°C (100% male), 27.5°C (50% male), and 30.5°C (0% male) without the experimental manipulation used by Rhen and Lang. Turtles from 21.5 and 30.5°C were smallest at hatching, but individuals from 21.5 and 24.5°C grew fastest and chose warmer microenvironments over ~10 months posthatching. This result held even after hibernation for 6 months at 7°C! Subsequent work indicated that males generally had higher resting metabolic rates than females, whereas the latter had higher blood levels of thyroxine than the former (O'Steen & Janzen 1999). The contradictory findings of these growth studies, along with comparative analyses, question the broad validity of the sexual size dimorphism hypothesis (Janzen & Paukstis 1991b; but see Ewert et al. 1994). Regardless, further experimental work in *Chelydra* and in other turtles with TSD is needed (Roosenburg & Kelley 1996; Freedberg et al. 2001), in particular, under natural or seminatural conditions (Janzen & Morjan 2002).

The only other experimental study to investigate the adaptive significance of TSD in *Chelydra* adopted a behavioral, performance-based approach recommended for microevolutionary research (sensu Arnold 1983). Eggs from Illinois were incubated at 26°C (100% male), 28°C (62% male), and 30°C (0% male) (Janzen 1995). Hatchlings were weighed, measured, and raced on land and in water in the laboratory to obtain measures of potentially fitness-related traits. Turtles were marked individually, sexed surgically, and then released into a fenced outdoor experimental pond. Survivorship after 6 months was assessed and evaluated in the context of the phenotypic traits measured at hatching. Turtles from the "single sex"–producing temperatures (26 and 30°C) were less likely to be active during performance trials in the laboratory, which translated into *higher* survivorship in the pond. The presumption was that these individuals exhibited a survival advantage over turtles from 28°C because common visual predators at the pond (e.g., bullfrogs) cue on movement of prey. Overall, the results of this study were interpreted as being consistent with the Charnov-Bull model. Although intriguing, this work has yet to be repeated in any other population or species and suffers from having investigated a relatively narrow portion of the thermal range of incubation temperatures.

The search for adaptive explanation for TSD in *Chelydra* and in other amniotes has been surprisingly frustrating given the success of the Charnov-Bull model for other taxa with environmental sex determination, including a fish with TSD (Conover 1984). Incubation temperature clearly influences neonatal and juvenile phenotypes in *Chelydra* and other species with TSD, including traits that might reasonably be construed to affect fitness (Shine 1999; see above). The critical issue now is not so much more work on the phenotypic effects of incubation temperature, but rather the great need for experimental tests that address possible changes in male-to-female fitness ratios across incubation temperatures (J. Bull, pers. commun.). Until then, we are left with a literature comprising plausible or partial stories consistent with, but not conclusive of, an adaptive explanation for TSD in "reptiles" à la the Charnov-Bull model.

Other models (e.g., Freedberg & Wade 2001) may instead prove to be more fruitful than the Charnov-Bull model in explaining the occurrence of TSD in amniotes. Even so, the fitness benefits of TSD over GSD need only be remarkably minimal in long-lived species to favor the evolution of TSD (Bull & Bulmer 1989), making identification of any adaptive basis for TSD in most amniotes a Sisyphean task. For that matter, TSD originated so long ago in turtles that the conditions for adaptation may no longer obtain, such that TSD may have spread by having been correlated with a different but adaptive trait (Janzen & Paukstis 1991a) or persists simply because it is not maladaptive (Janzen & Paukstis 1988; Girondot & Pieau 1999). Perhaps research on the many related issues raised in this review will shed light on the true explanation for TSD in amniotes.

FUTURE DIRECTIONS

In this review, I have discussed the major issues involving TSD in amniotes, highlighting the many important empirical contributions studies of *Chelydra* have made to a greater understanding of this unusual sex-determining mechanism. Nonetheless, much work remains to adequately evaluate many lingering unresolved questions concerning the basic biology of TSD. *Chelydra* clearly has a strong role to play in successfully tackling these challenges in the future. I see four thrusts of inquiry that are important, in particular, if we hope to develop a complete picture of the biological significance of TSD:

1. Relationships of fluctuating nest temperatures to offspring sex ratios—sophisticated statistical models to successfully predict offspring sex ratios from nest temperature traces. Snapping turtles lay especially deep nests that contain thermal gradients and thus will challenge our ability to construct general statistical models of this sort.

2. Mechanistic underpinnings—detailed studies to clarify the genetic factors and developmental pathways involved in sex determination and sexual differentiation. *Chelydra* can continue to play an important role in such studies because of availability, large clutch

sizes to assess among-clutch variation, and the presence of pattern II TSD.

3. Role(s) of maternal effects—investigations, especially field experiments, of yolk hormones, nesting phenology, and nest-site selection to better elucidate the adaptive significance of TSD. Among-clutch variation in yolk hormones and large population sizes place *Chelydra* in an excellent position to contribute strongly to maternal effects studies involving TSD.

4. Impacts of climate and habitat change—long-term field studies of offspring sex ratios and large-scale quantitative (and perhaps experimental) projects on nesting biology and reaction norms of sex ratio to address increasingly important microevolutionary and conservation issues. Again, large clutch sizes, population sizes, and geographic range position *Chelydra* to serve as an important model for such increasingly crucial research.

ACKNOWLEDGMENTS

My thanks to Gary Packard and Gary Paukstis for getting me started with *Chelydra* research many years ago. Rachel Bowden, Jim Bull, Carrie Morjan, and Tony Steyermark provided constructive comments on the manuscript and Mike Ewert helped with literature pointers and other suggestions. My work on aspects of *Chelydra* sex determination has been supported by National Science Foundation grants DDIG BSR-8914686, DEB-9629529, UMEB IBN-0080194, and LTREB DEB-0089680, and by numerous, generous Turtle Camp and Janzen Lab participants.